300 Rec'd PCT/PTO 08 OCT 1998

FORM PTO-1390 (REV. 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

(ATTORNEY'S DOCKET NUMBER 514413-3669

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

(U.S. APPLICATION NO. (If known, see 37 C.F.R.1.5) 09/155921

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	[EARLIEST] PRIORITY DATE CLAIMED
PCT/EP97/01741	08 APRIL 1997	11 APRIL 1996

TITLE OF INVENTION: **PROCESS FOR THE PRODUCTION OF PLANTS WITH ENHANCED GROWTH CHARACTERISTICS**

APPLICANTS FOR DO/EO/US: **Günter DONN, Peter ECKES, Hubert MULLNER, Genes DUDITS, Katalin PAULOVICS, and Attila FEHER**

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐has been transmitted by the International Bureau.
   c. ☐is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒A translation of the International Application into English (35 U.S.C. 371(c)(2)), including 0 sheets of formal drawings and a copy of the International Search Report.
7. ☒Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☒are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐have been transmitted by the International Bureau.
   c. ☐have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐have not been made and will not be made.
8. ☐A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
10. ☐The annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒A FIRST preliminary amendment.
    ☐A SECOND or SUBSEQUENT preliminary amendment.
14. ☐A substitute specification.
15. ☐A change of power of attorney and/or address letter.
16. ☒Other items or information:
    PCT/IPEA/416,409, PCT/IB/308
    PCT/RO/101 (7 pages)
    Check No. 3046 for $690.00

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DATE OF DEPOSIT October 8, 1998

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17. ☒The following fees are submitted: (CALCULATIONS /PTO USE ONLY

Basic National Fee (37 CFR 1.492(a)(1)-(5):

Search Report has been prepared by the EPO or JPO.....$690.00 ($930.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) $700.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))...$770.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37CFR 1.445(a)(2) paid to USPTO.....$1040.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT = ($690.00 /

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐20 ☐30 ($ / months from the earliest claimed priority date (37 CFR 1.492(e)).

8 Claims	/Number Filed /	Number Extra	/Rate	(
Total Claims	/ 8 - 20 = /	0	/X $14.00	($000.00 /
Independent Claims	/ 3 - 3 = /	0	/X $64.00	($000.00 /
Multiple dependent claim(s) (if applicable)			/+ $210.00	($ /

TOTAL OF ABOVE CALCULATIONS = ($690.00 /

Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28). (/

SUBTOTAL = ($690.00 /

Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)).+ (/ ($ /

TOTAL NATIONAL FEE = ($690.00 /

Fee for recording the enclosed assignments (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **$40.00** per property + (/ ($ 00.00 /

TOTAL FEES ENCLOSED = **($690.00** /

(Amount to be: / (refunded /$

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a. ☒Our check in the amount of $690.00 to cover the above fees is enclosed.

b. ☐Please charge my Deposit Account No. 50-0320 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0320**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

WILLIAM F. LAWRENCE
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Dated: **OCTOBER 8, 1998**

SIGNATURE

WILLIAM F. LAWRENCE
NAME

28,029
REGISTRATION NUMBER

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09/155921
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PATENT
514413-3669

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	DONN et al.
U.S. Serial No.	:	
Corresponding Int'l. App.	:	PCT/EP97/01741
International Filing Date	:	08 April 1997
Priority Dates Claimed	:	11 April 1996
For	:	PROCESS FOR THE PRODUCTION OF PLANTS WITH ENHANCED GROWTH CHARACTERISTICS

745 Fifth Avenue
New York, NY 10151
October 8, 1998

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PRELIMINARY AMENDMENT

Box PCT
Hon. Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Preliminary to the examination of this U.S. national phase application, please enter the following amendments:

IN THE CLAIMS:

Please cancel Claims 1 to 8 and replace with Claims 9 to 16:

--9. A process for the production of plants with improved growth characteristics which comprises the following steps:

a. transfer and integration of a DNA sequence coding for a prokaryotic asparagine synthetase in the plant genome

b. wherein said DNA sequence is linked to a regulatory sequence for the expression of said DNA and import of the asparagine synthetase into the chloroplasts and/or plastids of a plant cell and wherein said plant cell expresses the asparagine synthetase in its chloroplasts and/or plastids and

c. regeneration of intact and fertile plants from the transformed cells.

10. A plant cell wherein a prokaryotic ammonium specific asparagine synthetase is expressed in its chloroplasts and plastids.

11. A plant cell according to claim 10 which contains a gene construct which provides a reduced level of expression of endogenous glutamine synthetase activity.

12. A plant, seeds and propagation material containing cells in claim 10.

13. A gene construct comprising a gene encoding a prokaryotic ammonium specific asparagine synthetase operatively linked to a regulatory sequence for the expression of said DNA and import of the asparagine synthetase into the chloroplasts and/or plastids of a plant cell and wherein said plant cell expresses the asparagine synthetase in its chloroplasts and/or plastids.

14. A gene construct according to claim 13, wherein the asparagine synthetase gene is an E. coli asparagine synthetase gene with a chloroplastic leader peptide at its N-terminus.

15. A vector containing a gene construct according to claim 13 which gene construct comprises a sequence which encodes a chloroplastic leader peptide at its N-terminus.

16. A plant cell transformed with the gene construct according to claim 13 or with vector according to claim 15.--

REMARKS

The preliminary amendment is being filed to ensure that the amended sheets of claims that are enclosed with the application papers are in fact pending in the application.

Entry of this amendment and an early examination on the merits are respectfully solicited.

Respectfully submitted,

FROMMER LAWRENCE & HAUG LLP

Attorneys for Applicant

By _____

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TITLE OF THE INVENTION

Process for the production of plants with enhanced growth characteristics

RELATED APPLICATIONS

Reference is made to U.S. application Serial No. 08/465,526, filed June 5, 1995, as a division of U.S. application Serial No. 08/360,176, now U.S. Patent No. 5,545,819; each of these U.S. applications and U.S. Patent are hereby incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to: improving plant growth by expression of at least one bacterial asparagine synthetase in the chloroplast and/or plastid of cells of the plant; methods for so improving plant growth including introducing a nucleic acid molecule encoding the bacterial asparagine synthetase into the plant genome (e.g., into plant cells and culturing and/or regenerating the cells into the plants) wherein the nucleic acid molecule is operably linked to a nucleic acid molecule comprising regulatory sequences for expression and for import of the bacterial asparagine synthetase into the chloroplast and/or plastid; and, to plants having such improved growth.

Several documents are cited in the following text. Documents cited herein are hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

Nitrogen often is the rate-limiting element in plant growth. Most field crops have a fundamental dependence on inorganic nitrogenous fertilizer. Mineral fertilizers are a major source for ground water pollution. Therefore it would be beneficial if plants could utilize the existing nitrogen more efficiently.

Nitrogen is taken up by the plant as inorganic compounds, namely nitrate and ammonia. The majority of this nitrogen is assimilated into organic compounds like amino acids. The enzyme glutamine sythetase plays a major role since it catalyses the assimilation of ammonia into glutamine. Glutamine together with asparagines are the main transport forms of nitrogen in plants. As described in EP 511 979 the expression of a bacterial asparagines synthetases leads to improved growth characteristics which may be enhanced by the additional treatment of the plants with the herbicide glufosinate, a glutamine synthetase inhibitor. Whereas WO 95/09911 describes the production of a plant with improved agronomic or nutritional characteristics by over expression of one or several nitrogen/metabolism enzymes Applicants have now been able to find a quite different way to improve plant growth characteristics.

SUMMARY OF THE INVENTION

It has surprisingly be found that it is possible to improve plant growth capacities by the targeted expression of at least one bacterial asparagine synthetase in the chloroplast.

The present invention is directed to a process for the production of plants with improved growth characteristics which comprises the following steps:

- transfer and integration of a DNA sequence coding for a bacterial asparagine synthetases in the plant genome
- wherein said DNA sequence is linked to regulatory sequences which ensures expression of said gene in a plant cell and leading to the import of the derived protein into the chloroplast and/or plastids of said plant cells and
- regeneration of intact and fertile plants from the transformed cells.

According to instant invention the term improved growth characteristics is to be understood as encompassing enhanced or faster and more vigorous growth as well as more yield and/or earlier flowering. The process according to instant invention leads also to bigger or more reproductive organs as for example the seeds or bigger or more storage organs as for example tubers.

According to instant invention the bacterial asparagines synthetases may also be expressed directly in the chloroplast by integrating the gene directly into the genome of the chloroplast and/or plastids by for example the biolistic transformation procedure (see US Patent No. 5,451,513 incorporated herein by reference).

Therefore, the instant invention is also directed to a process for the production of plants with improved growth characteristics which comprises the following steps:

- transfer and integration of a DNA sequence coding for a bacterial asparagine synthetases into the genome of the chloroplast and/or plastids of a plant

cells,

- expression of said gene under the control of appropriate regulatory elements and
- regeneration of intact and fertile plants from the transformed cells.

Surprisingly, it was possible to enhance the growth improving effect even more by reducing the level of the glutamine synthetase expressed in the plant cell.

Accordingly, the instant invention is also directed to processes for the production of plant cells wherein said plant cells express a further gene construct which leads to a reduced level of its endogeneous glutamine synthetase activity.

A "DNA sequence", as the term is used herein, can mean a nucleic acid molecule, e.g., an isolated nucleic acid molecule; and, a "regulatory sequence", as the term is used herein, can mean a nucleic acid molecule which functions to regulate expression and/or import, e.g., import into a chloroplast and/or plastid.

Thus, the invention provides a plant cell containing DNA coding for prokaryotic, e.g., bacterial, asparagine synthetase, e.g., ammonium-specific asparagine synthetase, type A, operably linked to a regulatory sequence for expression of the DNA and import of the asparagine synthetase into the chloroplast and/or plastid of the cell, wherein the cell expresses the asparagine synthetase. Thus, the plant cell expresses the asparagine synthetase in its chloroplast and/or plastid. The plant cell can also contain a construct which provides reduced levels of expression of

endogenous glutamine synthetase, e.g., the endogenous gene therefor can be deleted or disrupted.

The invention further provides a method for increasing growth of a plant comprising: transforming a plant cell so that the cell contains DNA coding for prokaryotic, e.g., bacterial asparagine synthetase, e.g., ammonium-specific asparagine synthetase, type A, operably linked to a regulatory sequence for expression of the DNA and import of the asparagine synthetase into the chloroplast and/or plastid of the cell, wherein the cell expresses the asparagine synthetase (e.g., in its chloroplast and/or plastid); and regenerating the plant from the cell. The plant is preferably intact and fertile.

The plant cell in the method can also have the endogenous gene for glutamine synthetase deleted or disrupted, or otherwise expressed at a reduced level. Thus, the method can include transforming a plant cell to have a reduced level of expression of endogenous glutamine synthetase (e.g., by disrupting or deleting the gene therefor) and so that the cell contains DNA coding for prokaryotic, e.g., bacterial asparagine synthetase, e.g., ammonium-specific asparagine synthetase, type A, operably linked to a regulatory sequence for expression of the DNA and import of the asparagine synthetase into the chloroplast and/or plastid of the cell, wherein the cell expresses the asparagine synthetase (e.g., in its chloroplast and/or plastid); and regenerating the plant from the cell. The plant is preferably intact and fertile.

The methods can further comprise treating the plant with a glutamine synthetase

inhibitor.

The DNA coding for the asparagine synthetase can be from *E. coli*. However, from this disclosure, and the documents cited herein, and the knowledge in the art, one skilled in the art can ascertain other genes encoding asparagine synthetase, i.e., asn-A genes, from other microorganisms, e.g. by any routine procedure, for instance:

1. Ascertaining an asn-A gene product activity by routine assays for the asparagine synthetase type A with subsequent purification of the enzyme, e.g., according to Cedar & Schwartz 1969, J. Biol. Chem., 244, 4112-21 and 4122-4127, Humbert & Simoni, 1980, J. Bacteriol., 142, 212-220, and Reitzer & Magasanik, 1982, J. Bacteriol., 151, 1299-1313; see also Herrmann and Somerville, "Amino Acids, Biosynthesis And Genetic Regulation", pp. 137-145 (Addison-Wesley Pub. Co. 1993).
2. Production and purification of polyclonal antibodies against the asn-A gene product according to well-known immunological methods. And,
3. Screening of expression libraries of microorganisms with isolated antibodies against asparagine synthetase type A according to well-known molecular biological methods.

The above-described procedures make it clear that a skilled artisan can obtain asn-A gene sequences from other microorganisms by routine methods. Preferred asparagine synthetase utilizes ammonium ions as an amide donor for the production of asparagine; and thus, preferred DNA encodes such asparagine synthetase. Further, the regulatory sequence can be for a chloroplastic leader peptide; and, the

DNA coding for asparagine synthetase and the regulatory sequence can thus encode a prokaryotic asparagine synthetase, e.g., a bacterial asparagine synthetase such as *E. coli* asparagine synthetase, with a chloroplastic peptide at its N-terminal.

In the methods described herein, the growth of the plant is increased relative to non-transformed plants.

The invention further comprehends a plant, seeds, propagule or propagation material, from the foregoing methods, or containing the foregoing cells.

Additionally, the invention comprehends a gene construct comprising an isolated nucleic acid molecule encoding a prokaryotic, e.g., bacterial, asparagine synthetase, e.g., ammonium-specific asparagine synthetase, type A, operatively linked to a regulatory sequence active in plants for expression of the nucleic acid molecule and import of the asparagine synthetase into the chloroplast and/or plastid of cells of plants, e.g., a chloroplastic leader peptide; and therefore, in an embodiment the invention can provide a gene construct comprising an isolated nucleic acid molecule encoding a prokaryotic, e.g., bacterial such as *E. coli*, asparagine synthetase with a chloroplastic leader at its N-terminus. The invention also comprehends vectors containing the inventive gene constructs. The vector can be useful for transforming plant cells. Thus, the invention comprehends a plant cell transformed with the gene construct or vector, as well as plants, seeds, and propagules or propagation materials containing such cells.

And, the invention comprehends gene constructs and vectors for reducing endogenous glutamine synthetase expression, e.g., for inserting termination codons after regulatory sequences and prior to coding sequences, or for otherwise disrupting the gene for endogenous glutamine synthetase, as well as cells transformed with such gene constructs or vectors, and plants, seeds and propagules or propagation materials containing such cells.

These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

DETAILED DESCRIPTION

A preferred method of introducing the nucleic acid segments into plant cells is to infect plant cells with A. tumefacient carrying an inserted DNA construct. The nucleic acid segments or constructs can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of A. tumefaciens. The T-DNA is transmitted to plant cells upon infection by A. tumefaciens, and is stably integrated into the plant genome. Under appropriate conditions known in the art, the transformed cells develop further into plants.

The Agrobacterium strains customarily employed in the art of transformation are described, for example see especially US Patent No. 5,188,958 and EP 0 270 615 B1, incorporated herein by reference.

Ti plasmids contain two regions essential for the production of transformed cells. One of these, named transfer DNA (T DNA), induces tumour formation. The other,

termed virulent region, is essential for the introduction of the T DNA into plants. The transfer DNA region, which is transferred into the plant genome, can be increased in size by the insertion of the foreign nucleic acid sequence without its ability of transfer being affected. By removing the tumour-causing genes so that they no longer interfere the modified Ti plasmid ("disarmed Ti vector") can then be used as a vector for the transfer of the gene constructs of the invention into an appropriate microspores. In the binary system, to have infection, two plasmids are needed: a T-DNA containing plasmid and a vir plasmid (see especially EP 116718 B1 and EP 120 516 B1).

Besides transformation using Agrobacteria there are many other techniques for the introduction of DNA available. These techniques include, e.g. the protoplast transformation (see EP 164 575) the micro injection of DNA, the introduction of DNA via electroporation as well as biolistic methods and virus mediated infection. From the transformed cells applying suitable media and techniques whole plants can be regenerated (see McCormick et al. (1986) in Plant Cell Reports 5: 81-84). The regenerated plants may be preferably used to cross them with existing breeding lines to improve their growth characteristics as well.

The DNA constructs used in instant invention consist of a transcription initiation region and, under the control of the transcription initiation region, a DNA sequence to be transcribed. The DNA sequence may comprise a natural open reading frame including transcribed 5' and 3' flanking sequences. Alternatively, it may comprise an anti-sense sequence that encodes the complement of an RNA molecule or portion thereof (as described in EP 140 308 B1 and EP 223 399 B1) in order to suppress

the expression of the internally expressed glutamine synthetases.

The initiation regions may be used in a variety of contexts and in combination with a variety of sequences. The RNA coded sequences of a gene may be those of a natural gene, including the open reading frame for protein coding and frequently the 5' and 3' untranslated sequences. The RNA translational initiation sequences are included in the constructs, either from the promoter domain or from the attached coding sequences.

Attached to the above sequences are appropriate transcription termination and polyadenylation sequences.

The DNA constructs used in the transformation process according to instant invention may comprise sequences coding for naturally occurring or genetically modified transit peptides (see for example EP 189 707 B1).

Examples of additionally expressed sequences or genes to be expressed from the constructs of the subject invention include:

- especially antisense or sense genes (for gene suppression or cosuppression); as well as additionally
- nutritionally important proteins: growth promoting factors;
- yield enhancing genes or factors, e.g. an invertase gene, a citrate synthase, a polyphosphate kinase;
- proteins giving protection to the plant under certain environmental conditions, e.

g. proteins giving resistance to metal or other toxicity;

- stress related proteins giving tolerance to extremes of temperature, freezing, etc.
- proteins of specific commercial value;
- genes causing increased level of proteins, e. g., enzymes of metabolic pathways,
- genes causing increased levels of products of structural value to a plant host, e. g., herbicide resistance, fungus resistance, e.g. chitinase genes, glucanase genes, proteins synthesis inhibitor genes, ribosome inhibitory protein genes, viral resistance, e.g. ribozymes, virus coat protein genes.

The subject constructs will be prepared employing cloning vectors, where the sequences may be naturally occurring, mutated sequences, synthetic sequences, or combinations thereof. The cloning vectors are well known and comprise prokaryotic replication systems, markers for selection of transformed host cells, and restriction sites for insertion or substitution of sequences. For transcription and optimal expression, the DNA may be transformed into plant cells for integration into the genome, where the subject construct is joined to a marker for selection or is co-transformed with DNA encoding a marker for selection.

The selection of transformed cells is enabled by the use of a selectable marker gene which is also transferred. The expression of the marker gene confers a phenotypic trait that enables the selection. Examples for such genes are those coding for antibiotics or herbicide resistance, e.g. genes causing resistance against glutamine synthetases inhibitors, e.g. bialaphos or phosphinothricin resistance conferred by genes isolated from Streptomyces hygroscopicus or viridochromogenes (BAR/PAT). Other examples are the neomycin phosphotransferase or the glucuronidase gene.

The class of transgenic plants which are covered by this invention is generally as broad as the class of higher plants susceptible to transformation, including both monocotyledonous and dicotyledonous plants. It is known that theoretically all plants can be regenerated from cultured totipotent cells, including but not limited to all major cereal crop species, sugarcane, sugar beet, cotton, fruit and other trees, legumes and vegetables.

Examples of families that are of special interest are Poaceae, but also Solanaceae, Malvaceae and Brassicaceae.

Some suitable species include, for example, species from the genera Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Ciohorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hererocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Lolium, Zea, Triticum, Sorghum, and Datura.

Examples of species of commercial interest that can be protected include:

- tobacco, Nicotiana tabacum L.
- tomato, Lycopersicon esculentum Mill,
- potato, Solanum tuberosum L.,
- Canola/Rapeseed,
- Brassica napus L.,

- cabbage, broccoli, kale etc.,
- Brassica oleracea L.,
- mustards Brassica juncea L.,
- Brassica nigra L.,
- Sinapis alba L. (Brassicaceae),
- petunia, Petunia hybrida (Solanaceae)
- sugar beet, Beta vulgaris,(Chenopodiaceae),
- cucumber, Curcurbita sp. (Curcurbitaceae),
- cotton, Gossypium sp., (Malvaceae),
- sunflower, Helianthus annuus,
- lettuce Lactuca sativa, (Asteraceae=Compositae),
- pea, Pisum sativum,
- soybean, Glycine max and alfalfa, Medicago sp. (Fabaceae=Leguminoseae),
- asparagus, Asparagus officinalis;
- gladiolus, Gladiolus sp., (Lilaceae);
- corn, Zea mays;
- rice, Oryza sativa (Poaceae);
- wheat, Triticum aestivum (Poaceae); and
- barley, Hordeum vulgare (Poaceae).

In an preferred embodiment the invention covers transformed potato, tobacco, corn, sugar beet, cotton, rape seed, soy bean, lupine, rice and wheat. Especially preferred are potatoes

The invention additionally relates to transformed plants which have been

regenerated out of different cell types and which have been transformed according to instant invention.

The transformation can be carried out as described in the following examples, provicded by way of illustration only.

EXAMPLES

In general, preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, Southern blots, DNA ligation and bacterial transformation were carried out using standard methods. (Maniatis et al., Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory (1982), referred to herein as "Maniatis" and hereby incorporated by reference.)

Example 1:

Fusion of a bacterial asparagine synthetase gene to the nucleotide sequence for a duplicated chloroplast transit peptide

Based on the complete nucleotide sequence of the ASN-A gene from E. coli (Nakamura et al. (1981) or EP 511 979) the gene was cloned as a Hga 1 /Pst 1 fragment into the vector pUC18. By means of PCR based in vitro mutagenesis a SphI site was created at the ATG translational start codon changing the nucleotide sequence from AAA ATG AAA ACC GCT (SEQ ID No: 1) into GGC GCATG CAG AAA ACC GCT (SEQ ID No.: 2). This mutation introduced an additional codon for glutamic acid into the gene directly following the ATG translation start codon.

The nucleotide sequence for a modified transit peptide from the small subunit of Ribulosebisphosphat Carboxylase from pea was isolated from the vector pNi6/25 (Wasmann, C.C. et al (1986) Mol. Gen. Genet. 205: 446-453) as a Hind3/Sph1 fragment. This transit peptide contains a duplication of 20 amino acids compared to the natural transit peptide.

The sequence of the duplicated transit peptide and ASN-A gene were fused by ligating the Sph1 sites resulting in tpASN. The tpASN gene was exised as a Hind3/Pst1 fragment and after changing the Hind3 site into a Kpn1 site cloned between CaMV 35S promoter and -terminator of the vector pDH51 $^{\delta}$Kpn.

Example 2:

Expression of the tpASN gene in tobacco and rape seed

The 35S-promoter/tpASN gene/35S-terminator cassette from pDH51 $^{\delta}$Kpn was isolated as an EcoR1 fragment, Hind3 linkers were added and the fragment was cloned into the Hind3 site of the vector pHOE6/Ac, which confers phosphinothricin resistance to plants. The resulting vector was called pHOE6Ac/tpASN. This vector was transformed into the C58 Agrobacterium strain MP9ORK (Koncz et al., Mol. Gen. Gen., 204, 383-396 (1986)).

Tobacco and rape seed plants were transformed following published procedures. Plants were regenerated on Murashige and Skoog based media.

Transformed plants were selected because of their resistance to the herbicide

phosphinothricin (PPT). PPT resistant plants were analysed for the presence of the bacterial asparagine synthetase gene. In a Northern Blot analysis ASN-A specific RNA was detected in the plants. With polyclonal antibodies it is demonstrated that the protein was targeted into the chloroplasts.

Example 3:

Expression of the tpASN gene in maize

The 35S-promoter/tpASN gene/35S-terminator cassette from pDH51 δKpn was isolated as an EcoR1 fragment, Hind3 linkers were added and the fragment was cloned into the Hind3 site of the vector pB2/35SAc resulting in pB35SAc/tpASN. This vector was used to transform maize protoplasts according to published procedures (EP 511 979 or EP 164 575). Plants were regenerated on Murashige and Skoog based media. Transformed plants were selected because of their resistance to the herbicide phosphinothricin (PPT). PPT resistant plants were analysed for the presence of the bacterial asparagine synthetase gene. In a Northern Blot analysis ASN-A specific RNA was detected in the plants. With polyclonal antibodies it is demonstrated that the protein was targeted into the chloroplasts.

Example 4:

Inhibition of chloroplastic glutamine synthetase by expression of the antisense gene in tobacco and rape seed

The coding sequences for the chloroplastic isoenzymes of Nicotiana sylvestris and

Brassica napus were cloned by PCR methods from the genomic DNA of the respective plants. The resulting fragments were cloned as ApaI fragments in antisense orientation between 35S-promoter and -terminator from CaMV located on the vector pRT100. The 35S-promoter/GS-antisense/35S-terminator cassettes were isolated as Pst1 fragments and cloned into the Pst1 site of the vector pHOE6/AcK3. This vector was transformed into the C58 Agrobacterium strain MP9ORK (Koncz et al. supra (1986)). Tobacco and rape seed plants were transformed following published procedures. Plants were regenerated on Murashge and Skoog based media with reduced amounts of ammonia as described.

Transformed plants were selected because of their resistance to the herbicide phosphinothricin (PPT). PPT resistant plants were screened with Southern Blot hybridization for the presence of the ASN-A gene. Southern positive plants were analysed for the inactivation of the chloroplastic glutamine synthetase gene by Northern blots. Plants with the most reduced GS RNA level were selected.

Example 5:

Inhibition of chloroplastic glutamine synthetase by expression of the respective antisense gene in maize

The coding sequences for the chloroplastic isoenzymes of Zea mays, was cloned by PCR methods from the genomic DNA. The resulting fragment was cloned as ApaI fragment in antisense orientation between 35S-promoter and terminator from CaMV located on the vector pRT1OO. The 35S-promoter/GS-antisense/35S-terminator cassette was isolated as Pst1 fragment and cloned into the vector pB2/AcK3.

This vector was used to transform maize protoplasts according to published procedures. Plants were regenerated on Murashge and Skoog based media with reduced amounts of ammonia as described. Transformed plants were selected because of their resistance to the herbicide phosphinothricin (PPT). PPT resistant plants were screened with Southern Blot hybridization for the presence of the ASN-A gene. Southern positive plants were analysed for the inactivation of the chloroplastic glutamine synthetase gene by Northern blots. Plants with the most reduced GS RNA level were selected.

Example 6:

Asparagin content in transgenic asparagin synthetase expressing plants

Leaf material from wild type and different ransgenic asparagin synthetase expressing plants was homogenized in buffer. The extracts were run over a Biotronic amino acid analyser. Concentration of the amino acid asparagine were measured and are given in pmol/µl of extract.

	NT-WT	NT-TPASN-2	NT-TPASN-3	NT-TPASN-5	NT-TPASN-11
ASN	586,855	890,26	3338,5551	1506,6314	992,0319

The concentration of asparagine correlated with the expression of the asparagine synthetase gene as measured on Northern and Western Blots.

Example 7:

Production of transgenic potato lines carrying the bacterial asparagine synthetase gene

The above mentioned construct was used to transform potato plants (Solanum tuberosum L. cv. Desiree 25). The control, non-transformed plant material went through an in vitro regeneration process comparable to the transformants. The tuber tissues were transformed according to the process as described above using the Agrobacterium technology.

The presence of the bacterial asnA gene was proven by hybridization of genomic plant DNAs with a chimeric gene specific fragment. The experiments confirmed that the transformants expressed the transferred gene while the control plants lacked the enzyme.

Northern analysis was carried out by hybridization of total RNA from the transformed potato lines, the hybridization experiment indicated the presence of specific mRNA in the transformants whereas the control plant lines showed again no detectable signal.

Example 8:

Growth behaviour of transgenic maize and tobacco plants

Transgenic asparagine synthetase expressing plants and transgenic asparagine synthetase expressing plants with reduced glutamine synthetase activity were grown

side by side with wild type plants in the greenhouse. The transgenic plants showed a more vigorous growth and flowered earlier than wild type plants.

Field experiments with transgenic potato plants carrying the bacterial asparagine synthetase gene

Experiment A

Genotype	Tuber weight per plant (gram)	% of control
Control plant	135.0	100.0
Trans. Asl	168.6	124.0
Trans.As2	182.3	135.0

Experiment B

Genotype	Tuber weight per plot (kg)	% of control
Control Plant	8.16	100.0
Trans. Asl	11.39	139.5
Trans. As2	10.94	127.0

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

Article 34

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ANNEX

~~Substitute page~~

CLAIMS

1. A process for the production of plants with improved growth characteristics which comprises following steps:

- transfer and integration of a DNA sequence coding for a prokaryotic asparagine synthetase in the plant genome
- wherein said DNA sequence is linked to a regulatory sequence for the expression of said DNA and import of the asparagine synthetase into the chloroplasts and/or plastids of a plant cell and wherein said plant cell expresses the asparagine synthetase in its chloroplasts and/or plastids and
- regeneration of intact and fertile plants from the transformed cells.

2. A plant cell wherein a prokaryotic ammonium specific asparagine synthetase is expressed in its chloroplasts and plastids.

3. A plant cell according to claim 2 which contains a gene construct which provides a reduced level of expression of endogenous glutamine synthetase activity.

4. A plant, seeds and propagation material containing cells as claimed in claims 2 and 3.

5. A gene construct comprising a gene encoding a prokaryotic ammonium specific asparagine synthestase operatively linked to a regulatory sequence for the expression of said DNA and import of the asparagine synthetase into the chloroplasts and/or plastids of a plant cell and wherein said plant cell expresses the asparagine synthetase in its chloroplasts and/or plastids.

AMENDED SHEET

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6. A gene construct according to claim 5, wherein the asparagine synthetase gene is an E. coli asparagine Synthetase gene with a chloroplastic leader peptide at its N-terminus.

7. A vector containing a gene construct according to claims 5 and 6 which gene construct comprises a sequence which encodes a chloroplastic leader peptide at its N-terminus.

8. A plant cell transformed with the gene construct according to claims 5 and 6 or with vector according to claim 7.

AMENDED SHEET

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

As below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Process for the production of plants with enhanced growth characteristics

the specification of which
- ■ is attached hereto
- ■ was filed on April 8, 1997 as International Application PCT/EP97/01741

and including all the amendments through the date hereof.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s) for which Priority is Claimed:

European Application, 96 105 679.3 of April 11, 1996

And I hereby appoint
William F. Lawrence, Registration No. 28,029, of the firm FROMMER LAWRENCE & HAUG, LLP whose post office address is 745 Fifth Avenue, New York, New York 10151, or their duly appointed associate, my attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and specify that all communications about the application are to be directed to the following correspondence address:

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William F. Lawrence

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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